PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 19/34, C12Q 1/68 — A1

(11) International Publication Number: WO 93/03167

(43) International Publication Date: 18 February 1993 (18.02.93)

(21) International Application Number: PCT/US92/06477

(22) International Filing Date: 4 August 1992 (04.08.92)

(30) Priority data:
741,109 6 August 1991 (06.08.91) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; University of California at Los Angeles, 405 Hilgard, 1400 Ueberroth Building, Los Angeles, CA 90024 (US).

(72) Inventors: SIGMAN, David, S. ; 1220 N. Kenter Ave., Los Angeles, CA 90049 (US). SIMPSON, Larry ; 3520 Stoner Avenue, Los Angeles, CA 90066 (US). AVILA, Herbert ; 227 N. Kenter Ave., Los Angeles, CA 90049 (US).

(74) Agents: BERMAN, Charles; Sheldon & Mak, 10990 Wilshire Boulevard, Suite 440, Los Angeles, CA 90024 (US) et al.

(81) Designated States: BR, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD OF ISOLATION OF DNA

(57) Abstract

A rapid and efficient method of DNA isolation, storage, and cleavage that provides DNA suitable for amplification by a sequence-specific method is provided. The method for isolating biologically active DNA from a biological sample having DNA-containing structures includes: (1) contacting a biological sample containing DNA-containing structures with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse DNA-containing structures in the sample and a chelating agent to preserve the DNA from degradation to form a mixture of the biological sample and the lysis and storage buffer; (2) incubating the mixture formed in step (1) with a metal-containing chemical nuclease that cleaves the DNA to DNA fragments; and (3) purifying the DNA fragments. The DNA isolated can be catenated closed circular DNA, such as kinetoplast DNA of *Trypanosoma cruzi*. The purified DNA fragments can be used for amplification in a sequence-based DNA amplification system employing primers that hybridize to the DNA in order to determine the presence of a specific DNA sequence in the fragements. The combination of the isolation and amplification methods can be useful for the detection of parasitic, bacterial, and viral diseases by identification of DNA sequences associated with the organisms causing them.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

METHOD OF ISOLATION OF DNA

BACKGROUND

Increasingly, DNA-based diagnostic methods are coming into use for the diagnosis of infectious and parasitic diseases. Many of these techniques involve DNA amplification procedures, such as the polymerase chain reaction procedure (PCR), as described in United States Patents Nos. 4,683,195, 4,683,202, 4,800,159, and 4,965,188 to Mullis et al. However, such DNA-based amplification procedures require that DNA be isolated in a stable, biologically active form from blood or other biological fluids; optimal application of such amplification procedures further requires that the DNA to be amplified be present in the form of linear fragments of less than about 2 kb in length. Moreover, because there is often a considerable lapse of time between the collection of a blood specimen from a patient and the testing of DNA isolated from a blood sample, by DNA amplification procedures, there is a need for a method of storing DNA stably for periods of time in blood. There further exists a need for the conversion of the DNA in these biological samples, which may be in the form of closed circular catenanes or other interlinked forms, into linearized double-stranded DNA fragments suitable for amplification. This method must be rapid, easy to

perform, and capable of releasing the DNA efficiently from cells or other structures and cleaving it into fragments suitable for amplification.

The need for a method of isolation and storage of DNA is particularly acute in connection with the diagnosis of parasitic diseases by DNA amplification procedures. Many of these diseases, including Chagas' disease, leishmaniasis, and malaria, occur primarily in tropical areas in which medical facilities are relatively primitive and a substantial period of time may elapse between collection of the sample from a patient suspected of infection and its testing. Recently, considerable work has been directed towards diagnosis of these diseases by PCR, as described in Avila et al. (Mol. Biochem. Parasitol. 42:175 (1990)), Sturm et al. (Mol. Biochem. Parasitol. 33:205 (1989)), Rodgers et al. (Exp. Parasitol. 71:267 (1990)) and Moser et al. (J. Clin. Microbiol. 27:1477 (1989)). Additionally, Ashall et al. (J.Clin. Microbiol. 26:576 (1988)) describe the detection of Trypanosoma cruzi in mammalian blood by a DNA-DNA hybridization procedure employing radiolabeled total parasite DNA under conditions that favor hybridization of repetitive DNA sequences.

The use of high EDTA concentrations to isolate DNA has been described in Simpson & Berliner, "Isolation of the Kinetoplast DNA of Leishmania tarentolae in the

form of a Network," J. Protozool. 21:382-393 (1974). Zolg et al. (Am. J. Trop. Med. Hyg. 39:33 (1988)) describe a method for storage of blood samples to be tested for the presence of the parasite Plasmodium falciparum using specific DNA probes, in high salt lysates. The lysates are prepared using water to lyse the erythrocytes; a detergent/EDTA mix to lyse the parasites, followed by the addition of cesium trifluoroacetate. Disadvantages of this procedure include the necessity to lyse the erythrocytes first, the requirement for use of a detergent, and the expense of the cesium salt. In addition, this system may inhibit enzymes such as exonucleases added to the lysate to cleave DNA.

The need for a method to isolate and prevent degradation of DNA in blood samples from patients suspected of infection with parasites or other infectious agents during storage, and to prepare the DNA for amplification and/or hybridization procedures so that diagnosis of infection can be accomplished, has gone unmet.

SUMMARY

Accordingly, the present invention provides a rapid and efficient method of DNA isolation, storage, and cleavage that meets these needs and provides DNA suitable

for amplification by a sequence-specific method, such as PCR. In general, the method of the present invention for isolating biologically active DNA from a biological sample having DNA-containing structures comprises the steps of:

(1) contacting a biological sample containing DNA-containing structures with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse DNA-containing structures in the sample and a chelating agent to preserve the DNA from degradation to form a mixture of the biological sample and the lysis and storage buffer;

(2) incubating the mixture formed in step (a) with a metal-containing chemical nuclease that cleaves the DNA to form DNA fragments; and

(3) purifying the DNA fragments.

The DNA in the DNA-containing structures can be present in the form of catenated closed circles. It can be isolated from a parasite. In particular, the DNA can be isolated from a kinetoplastid parasite such as Trypanosoma cruzi. Alternatively, the DNA can be associated with a virus such as herpes simplex virus (HSV). RNA can also be isolated from RNA-containing structures, and DNA can be produced from the RNA using reverse transcriptase for amplification. The biological sample can be a fluid sample such as mammalian blood, including human blood.

The non-amphipathic chaotropic salt is preferably a guanidinium salt, such as guanidinium chloride or guanidinium thiocyanate. More preferably, the guanidinium salt is guanidinium chloride. Most preferably, the guanidinium chloride is present in at least about 3 molar concentration in the mixture of the biological sample and lysis and storage buffer.

The chelating agent is preferably ethylenediamine- tetraacetic acid (EDTA). Most preferably, the EDTA is present in at least about 0.1 molar concentration in the mixture of the biological sample and lysis and storage buffer.

The general method can further comprise the steps of:

(4) amplifying the DNA by a sequence-specific method employing at least two primers that hybridize to the DNA fragments; and

(5) detecting disease associated with the presence of the DNA in an organism by identifying the DNA.

The primers employed in the sequence-specific method can comprise at least two primers that hybridize to a genetic marker of the organism from which the biological sample was isolated for identification of the genetic marker.

The metal-containing chemical nuclease can be selected from 1,10-phenanthroline-copper complex, derivatives of ferrous EDTA, metalloporphyrins, or octahedral metal complexes of 4,7-diphenyl-1,10-phenanthroline. Preferably, the metal-containing chemical nuclease is the 1,10-phenanthroline-copper complex.

An application of the general method is a method for detecting a disease caused by a kinetoplastid parasite. This method comprises the steps of:

(1) contacting a biological sample from a patient suspected of having a disease caused by a kinetoplastid parasite, with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse cells containing catenated closed circular kinetoplast DNA in the sample and a chelating agent to preserve the DNA in the cells from degradation to form a mixture of the biological sample and the lysis and storage buffer;

(2) incubating the mixture obtained in step (1) with a metal-containing chemical nuclease to linearize catenated closed circular kinetoplast DNA to form kinetoplast DNA fragments;

(3) purifying the DNA fragments to form purified kinetoplast DNA fragments suitable for amplification;

(4) amplifying the purified kinetoplast DNA fragments by a sequence-specific method employing at least two primers capable of hybridizing to the linearized DNA to form amplified kinetoplast DNA; and

(5) detecting disease by identifying the presence of the amplified kinetoplast DNA corresponding to the primers and thus having sequences specific for kinetoplast DNA of the parasite.

When this method is used to detect Chagas' disease, caused by T. cruzi, the primers hybridize to conserved regions in T. cruzi kinetoplast minicircular DNA.

A preferred method according to the present invention for the detection of a disease caused by T. cruzi comprises:

(1) mixing a mammalian blood sample suspected of containing kinetoplastid DNA from T. cruzi with about an equal volume of lysis and storage buffer comprising 6 M guanidinium chloride and 0.2 M EDTA, pH 8.0, to form a mixture and storing the mixture at room temperature for at least 24 hours;

(2) incubating the mixture obtained in step (1) with the metal-containing chemical nuclease 1,10-phenanthroline-copper complex to cleave the kinetoplast DNA to form linearized kinetoplast DNA;

(3) purifying the linearized kinetoplast DNA to form purified linearized kinetoplast DNA by:

(a) deproteinization with a phenol-chloroform mixture;

(b) ethanol precipitation with a glycogen carrier; and

(c) filtration through a microconcentrator;

(4) amplifying the purified linearized kinetoplast DNA by a sequence-specific method employing at least two primers capable of hybridizing to conserved regions of the catenated closed circular kinetoplast DNA of T. cruzi; and

(5) detecting disease caused by T. cruzi by identifying the presence of amplified DNA corresponding to the primers and thus having sequences specific for catenated closed circular kinetoplast DNA of T. cruzi by hybridization to the primers. Typically, the linearized DNA comprises fragments of from about 1 kb to about 1.5 kb in length.

The isolation and storage method steps of the method of the present invention can be practiced separately from cleavage of the DNA. A method of isolating and storing DNA from a cell-containing biological sample according to the present invention comprises the steps of:

(1) contacting a biological sample containing DNA present in cells with a lysis and storage buffer in order to liberate the DNA from the cells, the lysis and storage buffer comprising:

(a) a concentration of a non-amphipathic chaotropic salt sufficient to lyse cells in the biological sample when the lysis buffer is contacted with the biological sample; and

(b) a concentration of a chelating agent sufficient to preserve the DNA from degradation when the lysis buffer is contacted with the biological sample; and

(2) storing the liberated DNA in the mixture at a temperature of below about 65°C.

Preferably, the storage temperature is below about 4°C in order to preserve the DNA sample for at least about one year.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results of testing the stability of plasmid DNA as described in Example 1, infra.

Figure 2 shows the results of cleavage of isolated Trypanosoma cruzi kinetoplast DNA by the 1,10-phenanthroline-copper chemical nuclease ($OP\text{-}Cu^{2+}$) as described in Example 2, infra.

Figure 3 shows the frequency of single strand nicks in OP-Cu^{2+} linearized minicircle DNA as described in Example 2, infra.

Figure 4 shows results of a sensitivity titration of OP-Cu^{2+}-cleaved DNA, as described in Example 4, infra.

Figure 5 shows results from a blood sample of a chronic chagasic patient analyzed for the presence of T. cruzi minicircle sequences, as described in Example 5, infra.

Figure 6 shows the results of PCR amplification of T. cruzi DNA isolated from triatomid bug feces as described in Example 5, infra.

DESCRIPTION

The present invention is a rapid and efficient method of storing, isolating, and purifying biologically active DNA from a biological sample having DNA-containing structures. The DNA-containing structures include cells and viral particles. The method is particularly useful for isolating parasitic DNA that is present in the form of catenated closed circles, but the method is not limited to use on such DNA. The method allows for storage of the DNA in a state suitable for subsequent

controlled degradation by a chemical nuclease in order to cleave the DNA to fragments of a size suitable for subsequent amplification. The DNA can then be rapidly purified to render it suitable for use in an amplification method such as PCR.

In general, the method comprises:

(1) contacting a biological sample containing DNA-containing structures with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse DNA-containing structures in the sample and a chelating agent to preserve the DNA from degradation and prevent coagulation of the blood to form a mixture of the biological sample and the lysis and storage buffer;

(2) incubating the mixture formed in step (1) with a metal-containing chemical nuclease that cleaves the DNA to DNA fragments; and

(3) purifying the DNA fragments.

The method is particularly adapted for, but is not limited to, detection of parasitic diseases caused by parasites having catenated closed circular kinetoplast DNA, such as Trypanosoma cruzi, the protozoan causing Chagas' disease; (Sturm et al., Mol. Biochem. Parasitol. 33:205 (1989); Avila et al., Mol. Biochem. Parasitol. 42:175 (1990); Moser et al., J. Clin. Microbiol. 27:1487 (1989); Ashall et al., J. Clin. Microbiol. 26:576 (1988)); Leishmania species, the protozoa causing

leishmaniasis (Rodgers et al., Exp. Parasitol. 71:267 (1990)), and Plasmodium falciparum, P. vivax, and P. malariae, the protozoa causing human malaria (Zolg et al., Am. J. Trop. Med. Hyg. 39:33 (1988)). In addition, it can be used for the detection of other infectious diseases and infectious agents such as the provirus of human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis B virus, herpesvirus, Epstein-Barr virus, and Toxoplasma gondii.

I. ISOLATION OF DNA

A. The Biological Sample

The method is suitable for use on any biological sample containing DNA. The method is particularly adapted for storage and isolation of DNA from mammalian blood, including human blood, but the method is suitable for use on other biological samples, such as insect feces, biopsy tissue, urine, sputum, and lymphatic fluid. The method can be used on any volume of fluid sample, from microliters to liters, as needed.

B. The Lysis and Storage Buffer

The biological sample is mixed with the lysis and storage buffer of the invention. The lysis and storage buffer comprises: (1) a concentration of a non-

amphipathic chaotropic salt sufficient to lyse cells in the sample and (2) a concentration of a chelating agent sufficient to preserve the DNA from degradation. A non-amphipathic chaotropic salt is a salt, other than a detergent having distinct polar and non-polar moieties, that disrupts non-covalent bonds, such as hydrogen bonds, salt links, hydrophobic interactions, and van der Waals interactions, that are primarily responsible for the maintenance of secondary, tertiary, and quaternary structure in proteins and nucleic acids.

The non-amphipathic chaotropic salt is preferably guanidinium chloride or a chemically related salt such as guanidinium thiocyanate; other chaotropic salts such as lithium bromide, potassium thiocyanate, or potassium iodine are also usable. Most preferably, the guanidinium chloride is present in at least 3 molar concentration in the mixture of the biological sample and the lysis and storage buffer.

The chelating agent is preferably EDTA, although other chelating agents, such as sodium citrate, can also be used. Most preferably, the EDTA is present in at least 0.1 molar concentration in the mixture of the biological sample and the lysis and storage buffer.

Typically, the biological sample is mixed with about an equal volume of the lysis and storage buffer

containing about 6 molar guanidinium chloride and about 0.2 molar EDTA, pH 8.0.

When the lysis and storage buffer is mixed with the biological sample, the DNA-containing structures, such as cells or viral particles, present in the biological sample are lysed essentially instantaneously and the DNA is liberated from the DNA-containing structures. The DNA is stable in the mixture of the biological sample and lysis and storage buffer and can be stored at a storage temperature below about 65°C. At a storage temperature of 37°C, DNA stored in a mixture of lysis and storage buffer and sample remains intact for at least a month. At 4°C or -20°C, the DNA is stable for at least one year. The DNA can be stored at this stage for subsequent cleavage with the chemical nuclease and purification according to the remainder of the process of the present invention, or may be used in other techniques, such as nick translation to produce hybridization probes.

When the biological sample is blood, it is preferred to store the mixture of blood and lysis and storage buffer for an initial period of about 24 hours at room temperature (i.e., about 20-25°C), followed by subsequent storage at 4°C. It is not necessary to use freshly drawn blood; blood stored for periods of up to about 1 week can be used. In one version of the

procedure, blood is drawn into tubes containing anticoagulants such as citrate and/or heparin and then used after a period of storage of about 24 hours.

C. The Chemical Nuclease

The DNA in the lysis and storage buffer is fragmented by incubation of the biological sample-lysis and storage buffer mixture with a metal-containing chemical nuclease capable of nicking DNA by oxidative attack on the deoxyribose moiety (Sigman & Chen, Annu. Rev. Biochem. 59:207 (1990)). The chemical nuclease can be a 1,10-phenanthroline-copper complex (Sigman, Biochemistry 29:9097 (1990)); a derivative of ferrous EDTA such as methidiumpropyl-EDTA-iron (Hertzberg & Devan, J. Am Chem. Soc. 104:313 (1982); Schultz et al., J. Am. Chem. Soc. 104:6861 (1982); Tullius & Dombroski, Proc. Natl. Acad. Sci. U.S.A. 83:5469 (1986)); metalloporphyrins (Ward et al., Biochemistry 25:6875 (1986); Lee Doan et al., Biochemistry 25:6376 (1986); Groves & Farrell, J. Am. Chem. Soc. 111:4998 (1989)) or metal-containing octahedral complexes of 4,7-diphenyl-1,10-phenanthroline such as tris (4,7-diphenyl-1,10-phenanthroline) ruthenium (Barton, Science 233:727 (1986)). Preferably, the chemical nuclease is a 1,10-phenanthroline-copper complex, because it is not sensitive to inhibition by buffer components. In addition, the 1,10-phenanthroline-copper complex is

inexpensive and its reaction can be efficiently quenched by chelating agents in order to control the extent of cleavage. Other chemical nucleases, such as ferrous-EDTA, methidiumpropyl-EDTA-iron, metalloporphyrins, and metal-containing octahedral complexes of 4,7-diphenyl-1,10-phenanthroline, may be useful provided they are not substantially inhibited by the buffer.

The phenanthroline-copper chemical nuclease reagent introduces random single strand nicks into duplex DNA in the presence of peroxide (Sigman & Chen, Annu. Rev. Biochem. 59:207 (1990)). Because, on the average, one double strand cleavage occurs after ten random single strand breaks are introduced into a DNA molecule, this nuclease can be used to digest catenated kinetoplast DNA to linearize minicircles.

The chemical nuclease reaction using the 1,10-phenanthroline-copper complex is typically performed as follows: to one volume of the mixture of the biological sample and lysis and storage buffer containing 3 molar guanidinium chloride and 0.1 molar EDTA are added 0.1 volume each of 1 molar $MgCl_2$, 200 millimolar $CuSO_4$, 20 millimolar 1,10-phenanthroline and 7.5% H_2O_2 (freshly diluted from 30% stock solution). The reaction is initiated by addition of 0.1 volume of 3-mercaptopropionic acid and digestion of DNA is allowed to proceed for 30 minutes at 37° C. The reaction is stopped

by addition of 0.1 volume of 1.5 molar 2,9-dimethyl-1,10-phenanthroline. Reactions with other chemical nucleases are carried out as specified in the literature describing them. The cleavage process cleaves DNA to fragments that can be used in an amplification process. These fragments are linearized and are typically of about 1 to about 1.5 kb length.

The 1,10-phenanthroline-copper complex is believed to generate a highly reactive oxidative species and to react with the DNA through the formation of a reversible complex between the phenanthroline-copper reagent and the DNA. The reaction produces the following stable products: 5'-phosphorylated-termini, 3'-phosphorylated-termini, free bases and 5-methylenefuranone, as well as minor amounts of 3'-phosphoglycolate termini. The predominant reaction involves initial oxidative attack at the C-1 hydrogen of the deoxyribose by the DNA-bound coordination complex. Oxidative reaction is initiated within the minor groove of the DNA and the reagent exhibits preferential reactivity for DNA in the B form of the helix relative to DNA in the A form of the helix. The reaction is not specific for the nucleotide at the site of scission, but its rate does depend on local sequence. The most important influence on the intensity of cutting by the phenanthroline-copper complex at any sequence position is the neighboring 5'-nucleotide. Because the reaction can

be terminated at any time by the addition of a chelating agent with high affinity for copper, such as 2,9-dimethyl-1,10-phenanthroline, 2,9-dimethyl-4,7-phenanthroline, or EDTA, compensations can be made for any variations in reactivity due to local sequence. Preferably, the chelating agent used to quench the reaction is 2,9-dimethyl-1,10-phenanthroline. Thus, the cleavage method described herein is essentially independent of the DNA sequence, and the size of single-stranded DNA fragments produced by cleavage using the nuclease may be effectively controlled by regulating the time of digestion using the chelating agent.

In particular, cleavage with the 1,10-phenanthroline-copper complex of T. cruzi kinetoplast DNA, which is originally present in the form of closed circular catenated minicircles, results in individual linear DNA molecules of about 1.4 kb in length.

D. DNA Purification

The DNA is preferably purified to remove substances that can interfere with subsequent primer-based DNA amplification. Purification typically includes: (1) deproteinization; (2) precipitation of the DNA; and (3) filtration. The product of purification is suitable for amplification by PCR or another primer-based amplification step.

The deproteinization of the sample is typically performed by extraction with phenol or a 1:1 mixture of phenol and chloroform, which denatures proteins and separates proteins from nucleic acid. Preferably, the deproteinization is performed with extraction with a 1:1 mixture of phenol and chloroform.

Precipitation of the DNA is typically conducted using ethanol in the presence of a glycogen carrier and is preferably carried out in the presence of about 80 μg/ml glycogen and 0.3 molar sodium acetate at room temperature.

The final purification step can be performed by filtration through a microconcentrator. A suitable microconcentrator is a Centricon-100 microconcentrator manufactured by Amicon (Beverly, Massachusetts).

Subsequent to purification, the isolated and purified DNA can be amplified in a system, as described below, or can be used for other purposes for which highly purified DNA fragments are used, such as the generation of hybridization probes or incorporation into cloning vectors.

II. AMPLIFICATION OF ISOLATED DNA

The purified and fragmented DNA can be amplified by a sequence-specific method employing at least two oligonucleotide primers that hybridize to the DNA fragments. Such methods include, but are not limited to, the polymerase chain reaction (PCR method) of Mullis et al. as described in United States Patents 4,683,195, 4,683,202, 4,800,159, and 4,965,188 to Mullis et al., all of which are herein incorporated in their entirety by this reference. Preferably, the PCR method is performed using a thermostable DNA polymerase such as the Thermus aquaticus polymerase Taq I described in U.S. Patent Number 4,889,818 to Gelfand et al., and incorporated herein by this reference. Further details on the PCR method are given in PCR Protocols (M.A. Innis et al., eds., Academic Press (1989)), incorporated herein by this reference.

Other sequence-specific nucleic acid amplification systems employing primers are also known, such as the transcription-based amplification system of Gingeras et al. described in European Patent Application No. 368906, and the similar system of Davey and Malek described in European Patent Application No. 329822, both incorporated herein by this reference. These systems make use of an alternating cycle of amplification as DNA and RNA employing a primer incorporating a promoter, a

DNA-dependent RNA polymerase such as bacteriophage T7 RNA polymerase, and a RNA-dependent DNA polymerase or reverse transcriptase.

In any of these methods, the product of the amplification is a discrete linear DNA fragment or series of discrete DNA fragments that can be separated by electrophoresis on an agarose gel and identified by hybridization methods such as "Southern blot" hybridization following transfer of the DNA to nitrocellulose filters or other filters. Useful hybridization procedures are described in E.M. Sutter, "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis," J. Mol. Biol. 98: 503 (1975), incorporated herein by this reference. The hybridization is typically carried out with radioactively labeled DNA oligonucleotide probes; this serves to identify specific sequences originally present in the DNA used for amplification. If the probes are comprised of DNA associated with a disease-causing agent, such as a pathogenic bacterium, a DNA virus, or the DNA provirus of a RNA virus, the hybridization procedure can be used to identify DNA sequences associated with the disease-causing agent in the original sample and thus the presence of the disease.

III. APPLICATIONS OF THE METHOD

A. Diagnosis Of Parasitic Diseases

The DNA storage, cleavage, purification, and amplification method of the present invention is useful for the detection of parasitic diseases, particularly diseases caused by kinetoplastid trypanosomes.

These diseases include Chagas' disease, a major health problem in Latin America caused by Trypanosoma cruzi. Current serological and xenodiagnostic methods for the diagnosis of Chagas' disease are unreliable, especially in the case of chronic Chagas' disease in which the parasites are detectable within the blood only with difficulty, if at all, because of the low concentration of parasites within the blood.

T. cruzi kinetoplasts, which are DNA-containing structures in the mitochondria of the cells, contain DNA in the form of catenated minicircles, that, when cleaved by the chemical nuclease and purified according to the method of the present invention, can be efficiently amplified by the PCR process or another sequence-specific primer-based amplification process. Oligonucleotide primers specific to conserved regions in T. cruzi kinetoplast minicircles (kDNA) are used (Degrave et al., Mol. Biochem. Parasitol. 27:63-70 (1988); Sturm et al.,

Mol. Biochem. Parasitol. 33:205 (1989); Avila et al., Mol. Biol. Parasitol. 42:175 (1990)). These primers may be synthesized and are also commercially available from AMAC, Inc. (a Division of Genset, located in Westbrook, ME).

This results in a set of amplified DNA fragments that are species-dependent and strain-independent and can be used to detect the presence of T. cruzi DNA in the blood of patients, as well as in biological samples obtained from infected insect vectors or infected mammals such as mice.

In general, DNA isolated and amplified as described herein may be used to detect disease associated with the presence of the DNA using standard methods, such as by DNA hybridization using radioactively labeled probes to bind to specific sequences in the DNA (Sturm et al., Mol. Biochem. Parasitol. 33:205 (1989) and Avila et al., Mol. Biochem. Parasitol. 42:175 (1990)).

B. Other Applications and Advantages

The method of the present invention is not limited to storage, purification or detection of parasitic DNA or to minicircular DNA. Because the chemical nuclease that is employed typically cleaves DNA into linear fragments of from about 1 kb to about 1.5 kb

in length, an optimal size for amplification with primers of less than 1000 bases as required for PCR, the lysis, storage, cleavage, and purification process of the present invention can be used to detect DNA specific for any biological entity of interest from any biological sample, including blood, urine, sputum, or lymphatic fluid. The DNA detected can be from any etiological agent, including the DNA of parasitic protozoans, bacteria, or viruses such as herpes viruses, cytomegalovirus (CMV), hepatitis B virus, herpes virus, Epstein-Barr virus, or Toxoplasma gondii. Cellular DNA can also be detected, including mammalian or human DNA, and retroviral DNA such as the DNA of human immunodeficiency virus (HIV) associated with AIDS. The amplification allows the detection of extremely small quantities of DNA specific to a etiological agent, and thus can be used to detect the presence of the disease caused by the agent earlier than conventional serological diagnostic procedures.

When whole blood is used for the isolation of DNA according to the method of the present invention, genetic markers of the host species (animal or human) can be assayed in the same multiplex PCR assay in addition to genetic markers of parasites or other etiological agents of diseases. For example, in the case of humans, the genetic markers assayed can include a family of immune response genes such as the major histocompatibility locus

(MHC) genes which have been shown to be correlated with resistance to various diseases and susceptibility to autoimmune diseases. Other appropriate genetic loci for assay in multiplex PCR include the human homologues of those genetic markers found in animal models to confer resistance to parasitic diseases such as those caused by Leishmania or Trypanosoma cruzi. Any desired genetic locus, such as oncogenes and anti-oncogenes or genes involved in genetic diseases can be targeted for selective amplification during this assay, using the appropriate selection of primers and the isolated DNA.

RNA can also be isolated from RNA-containing structures such as cells or viruses and stored in a mixture of the sample and the lysis and storage buffer. The RNA can then be transcribed by reverse transcriptase to generate a RNA-DNA hybrid. The RNA in the hybrid is then degraded by the enzyme ribonuclease H specific for the RNA strand of a double-stranded RNA-DNA hybrid, and the resulting DNA strand made double-stranded by hybridization of a suitable primer and elongation of the primer by DNA polymerase.

An additional advantage of the method of the invention is that the use of the lysis and storage buffer and chemical nuclease of the invention can contribute to reduction of the infectivity of endogenous infectious organisms such as viruses or bacteria present in the

biological sample, because the infectivity is substantially destroyed by lysis of the bacterial cells or virus particles and also by cleavage of the viral nucleic acid. Treatment of membrane enveloped viruses with the 1,10-phenanthroline-copper chemical nuclease destroys their infectivity (Lembech et al., Fed. Proc. 44:1072 (1985).

The invention is illustrated by the following examples. The examples are for illustrative purposes only and are not to be construed as limiting the scope of the invention in any manner.

Example 1

Storage Of DNA In Guanidinium Chloride-EDTA Buffer

Human intravenous blood was freshly drawn and collected into tubes containing an equal volume of 2 X GE lysis and storage buffer (6 M guanidinium chloride, 0.2 M EDTA, pH 8.0). The resulting GEB (guanidinium/EDTA/blood) lysate was stored at 25°C for at least 24 hours and subsequently at 4°C for up to 6 months.

To test the stability of DNA in GEB lysates, plasmid DNA, pGEM 7Z (Promega, Madison, Wisconsin), was added to 2 tubes of GEB lysates. Each tube was stored at either 37°C or 65°C. Equivalent aliquots from each tube

were taken at different time intervals for up to four weeks. The aliquots were extracted once with phenol/chloroform (1:1, v/v) and ethanol-precipitated. The isolated DNA was electrophoresed in a 1% agarose gel to determine the percentage of nicked or linearized plasmid DNA, which can be detected by its relatively rapid mobility through the gel in contrast to intact catenanes, which remain at the top of the gel.

The results are shown in Figure 1; the electrophoretic pattern resulting from incubation at 37°C is designated (1A) and the pattern resulting from incubation at 65°C is designated (1B). The control lane is unincubated plasmid DNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE). Figure 1 shows that the DNA remains intact at 37°C for at least a month, with no apparent nicking or degradation. At 65°C, the DNA is nicked after a two-week incubation, as indicated by the disappearance of the closed circular DNA band and the increase in the nicked circular band. Even at 65°C, at least 50% of the DNA remained in the nicked circular or linear form after one week incubation. At 4°C or -20°C, DNA was shown to be stable in GEB lysate for at least one year. These results clearly demonstrate that the guanidinium chloride-EDTA reagent is a suitable medium for the lysis of cells contained in blood and the preservation of DNA at room temperature for a substantial period of time.

Example 2

Cleavage Of Kinetoplast DNA Using 1,10-Phenanthroline-Copper Complex

To 0.5 ml GEB lysate containing kinetoplast DNA, 0.05 ml of each of the following solutions was added: 1 M $MgCl_2$, 200 mM $CuSO_4$, 20 mM 1,10-phenanthroline, and 7.5% H_2O_2 (diluted fresh from 30% stock). The reaction was initiated by addition of 0.05 ml of 58 mM 3-mercaptopropionic acid. Digestion of DNA was allowed to proceed for 30 minutes or 60 minutes at 37°C. The reaction was stopped by addition of 0.05 ml of 1.5 M 2,9-dimethyl-1,10-phenanthroline. For GEB lysates containing T. cruzi kinetoplast DNA digestion was carried out for 60 minutes at 37°C, and aliquots were removed every 10 minutes. The reaction was quenched by addition of 2,9-dimethyl-1,10-phenanthroline. The aliquots were deproteinized with phenol/chloroform (1:1) and precipitated with ethanol. The DNA was denatured in glyoxal/DMSO and loaded onto a 1% agarose gel and electrophoresed as described by McMaster and Carmichael (Proc. Natl. Acad. Sci. U.S.A. 74:4835 (1977)). The gel was blotted and the DNA transferred to a Nytran filter (Schuell and Schuster, Keene, New Hampshire). Total T. cruzi kinetoplast DNA was nick-translated with ^{32}P-ATP and used as a hybridization probe. Nick-translation and hybridization conditions were as described in Simpson et

al., Nucl. Acids Res. 13:9577 (1985)), incorporated by reference herein.

The extent of cleavage of purified T. cruzi kinetoplast DNA in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by the phenanthroline-copper chemical nuclease reagent was monitored by agarose gel electrophoresis on a 1% agarose gel as a function of digestion time (Figure 2). In Figure 2, the M lane shows Hind III fragments of bacteriophage λ DNA and Hae III fragments of bacteriophage ϕX174 RF DNA as size markers. The disappearance of the kDNA from the well and the appearance of the 1.4-kb linear minicircle band as a function of digestion time is seen. After 10 minutes of incubation, the DNA was completely cleaved to minicircles.

Undigested catenated kinetoplast DNA remained in the well because, as a result of its large size, it was unable to enter the gel. After one minute of digestion, no catenated kinetoplast DNA could be detected in the well and the released linearized minicircle DNA migrated as a 1.4 kb band (Figure 2). Continued digestion led to the appearance of a lower molecular weight smear resulting from multiple cleavages per minicircle molecule. However, the reaction could be terminated at any point by the addition of a copper

chelating agent, such as 2,9-dimethyl-1,10-phenanthroline.

If the duplex minicircle DNA fragments released from the catenated network contain excessive single strand nicks, the DNA will not be an adequate substrate for PCR amplification. Upon denaturation, the size of the single-stranded fragment would have to be at least equal to the distance between the two PCR primers in order to obtain successful amplification. As described above, the DNA was cleaved with the phenanthroline-copper chemical nuclease in GEB lysate for increasing periods of time and the cleaved DNA was electrophoresed in a denaturing glyoxal gel to determine the size distribution of single-stranded fragments. The gel was blotted onto a nylon membrane and hybridized with ^{32}P-labeled T. cruzi kinetoplast DNA (kDNA). At time 0, a few decatenated minicircles undergoing replication can be seen as a 1.4-kb band in the gel. After cleavage, the minicircles were released from the kDNA networks as double stranded linearized molecules. By denaturing the linearized minicircles, it can be seen that the minicircles were increasingly nicked as a function of digestion time. Figure 3 shows that with increasing incubation time there was a decrease in the size of the single-stranded fragments caused by nicking. However, after 30 minutes, 90% of the minicircle fragments were larger than 310 bases. After 60 minutes, 50% of the fragments were

larger than 310 bases, and 80% of the fragments were larger than 118 bases. An incubation time of 30 minutes was selected for routine phenanthroline-copper digestion of blood lysates, at which time approximately 90% of the single-stranded fragments were longer than 310 bases, and would be appropriate amplification target molecules for the three sets of PCR primers which yield products of 83 bp, 122 bp, and 330 bp respectively (Sturm et al., supra). A control experiment showed the kinetoplast DNA digested with the phenanthroline-copper reagent in GEB lysate under standard conditions for 30 minutes was a suitable template for PCR amplification.

Example 3

Isolation Of Cleaved Minicircle DNA

After GEB lysates of T. cruzi were digested with the phenanthroline-copper reagent (OP-Cu^{2+}) for 30 minutes and the reaction quenched as described above, 500 µl aliquots containing decreasing numbers of minicircle molecules (calculated from the DNA concentration) were removed. Each 500 µl aliquot was extracted once with 100 µl phenol/chloroform (1:1). The aqueous phase was transferred to an Eppendorf tube containing 40 µg of glycogen (2µl of 20 mg/ml stock solution) and 50 µl of 3 M NaOAc. One milliliter of ethanol was added and the DNA was precipitated at room temperature by spinning the tubes in a microcentrifuge for 20 minutes.

The pellet was resuspended in 1 ml of water and transferred to a Centricon-100 microconcentrator (Amicon) containing 1 ml of water. The microconcentrator unit was centrifuged at 1000 x g in a clinical centrifuge for 10 minutes. The retentate was washed a second time with 2 ml of water. After the second 10 minute centrifugation 100 µl of concentrated retentate was collected as described by the manufacture of the microconcentrator. The retentate was used for PCR amplification as described below.

Example 4

Polymerase Chain Reaction Amplification Of Cleaved Minicircle DNA

70 µl of the retentate material was amplified in a 100 µl PCR reaction. The reaction conditions were as follows: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml BSA, 3 units of Taq DNA polymerase (Perkin-Elmer Cetus) and 100 picomoles of each primer. The primers used were three sets of primers specific to the four conserved regions in T. cruzi minicircles yielding the 330 bp minicircle variable and conserved region DNA fragment and the 83 bp and 122 bp conserved region fragments respectively, as described by Sturm et al., Mol. Biochem. Parasitol. 33:205 (1989) and Avila et al., Mol. Biochem. Parasitol. 42:175 (1990), both of which are incorporated by reference herein. The combination of the

26 base primer S33A and the 26 base primer S34A resulted in the generation of the 83 bp conserved region fragment. The combination of S34A and the 26 base primer S67 resulted in the generation of the 122 bp conserved region fragment. The combination of the 26 base primer S35 and the 21 base primer S36 resulted in the generation of the 330 bp variable region fragment (Sturm et al., supra). These primers are commercially available as Genset from Amac, Inc., Westbrook, Maine. The cycling profile was as follows: Denaturation, annealing, and elongation were done at 94°C, 60°C, and 72°C, respectively; each step was allowed to proceed for one minute for a total of 30 cycles. A 15 μl aliquot from each reaction was analyzed on agarose/Nusieve (FMC, Rockland, Maine) gels.

To determine the sensitivity of the PCR-minicircle DNA assay, the equivalent of 20 T. cruzi kinetoplast DNA catenated networks were added to 10 ml GEB lysate. The network DNA was cleaved with phenanthroline-copper as described in Example 2. After cleavage, the GEB lysate was diluted with kinetoplast DNA-free GEB lysate to give DNA concentrations of from 1 to 10,000 minicircles for 500 μl of GEB lysate. Total DNA was isolated from these aliquots as described in Example 3 and subjected to PCR amplification; 1/6 of each PCR reaction was loaded onto a gel. In all cases, the M lane shows Hae III fragments of bacteriophage φX174 RF DNA as size markers. In some cases, results are shown

from hybridization with a radioactively labeled probe after blotting of the gel as described in Example 2.

Figure 4A shows results from amplification of 83-bp fragments using 35 PCR cycles; the products were electrophoresed on a 2% agarose/3% Nusieve gel. The rapidly migrating band present in all lanes represents PCR primers or primer dimers.

Figure 4B shows results from amplification of 122 bp fragments using 30 PCR cycles; the products were electrophoresed on a 1% agarose/3% Nusieve gel. The top panel shows the stained gel; the low molecular weight band present in all lanes represents PCR primers. The bottom panel shows the hybridization of the blot with ^{32}P-labeled S34A oligonucleotide internal probe (Sturm et al., supra).

Figure 4C shows results from amplification of 330 bp variable and conserved region fragments with 30 PCR cycles; the products were electrophoresed on a 1% agarose/3% Nusieve gel. The top panel shows the stained gel. The bottom panel shows results from hybridization of the blot with ^{32}P-labeled S67 oligonucleotide internal probe (Sturm et al., supra; Avila et al., Mol. Biochem. Parasitol. 42:175 (1990)). The C1 and C2 lanes show 500 μl of undigested GEB lysate sample containing 10 kDNA

networks per 30 ml processed for PCR amplification as described for all other samples.

As shown by the electrophoresis patterns of Figure 4, at least 100 minicircles in 500 µl of GEB lysate were detected, either by ethidium bromide staining (83 bp PCR product) or by hybridization to a ^{32}P-labeled oligonucleotide probe (122 bp and 330 bp PCR products). Therefore this method detects the equivalent of 1% of the minicircle content of a single T. cruzi cell in 500 µl of phenanthroline-copper digested GEB lysate. These results indicate that the method can be used for the detection of a single T. cruzi cell in 20 ml of blood.

Example 5

Diagnosis of Chagas' Disease

A 49 year old female patient from Ecuador showing cardiac disturbances tested positive for T. cruzi in two different serological tests -- xenodiagnosis was negative. The serological tests employed were complement fixation (Sommerwirth and Jarett, Gradwohl's Clinical Laboratory Methods and Diagnosis (C.V. Mosby, St. Louis, 1980)) and ELISA (Goldsmith & Heyneman, Tropical Medicine and Parasitology (1989)). Xenodiagnosis was performed by the following procedure (E.L. Segura, Xenodiagnosis. In Chagas' Disease Vectors (Brenner and Stoka, eds., CRC

Press, Boca Raton, Florida, 1987), vol. II, pp. 41-45): Uninfected triatomids of several species, including Triatoma dimidiata, T. protracta and Rhodnius prolixus, were allowed to feed upon the patient. Second, third, fourth, and fifth nymphal as well as adult stages were used. Approximately one-third of the bugs fed, and defecation spots were found on the patient. Microscopic examination of bug feces at 30 and 60 days after feeding revealed no T. cruzi.

A 10 ml sample of venous blood from the patient was obtained and stored as GEB lysate. Phenanthroline-copper cleavage of the lysate was performed and DNA was isolated from two 500 μl aliquots and PCR amplified as described in Examples 2 through 4, supra.

Figure 5 shows a specific amplification of T. cruzi minicircle sequences from patient blood with two different sets of PCR primers. Figure 5A shows the amplification of an 83 bp fragment; the products are analyzed on a 1% agarose/3% Nusieve gel. The negative control in lane 1 is a GEB sample from a non-chagasic donor. The negative control in lane 2 is a sample lacking kDNA in the PCR reaction. The M lane shows DNA Hae III fragments of ϕX174 RF DNA as size markers. Figure 5B shows the amplification of 330 bp variable and conserved region fragments, run on a 2% agarose gel, blotted and hybridized with ^{32}P-labeled S67

oligonucleotide internal probe (Sturm et al., supra; Avila et al., supra.) A positive control is 100 fg gel-isolated OP-Cu^{2+}-cleaved kDNA; the negative controls are the same as (A).

Blood samples from four additional serology-positive chronic chagasic patients (three patients were xenodiagnosis-negative and one xenodiagnosis-positive) also tested positive for T. cruzi parasites by PCR amplification of minicircle DNA using the standard procedure of Examples 1-4. These results indicate that the method should prove useful in diagnosis of chronic Chagas' disease.

The DNA storage, cleavage, purification, and amplification method was extended to an insect vector and to biopsy material from infected mice. The abdominal contents of two T. dimidiata and two R. prolixus were collected and stored in GE buffer. The samples were processed as described in Examples 1-4. Figure 6 shows a specific amplification of kinetoplast DNA minicircle sequences from the insect abdominal contents. Minicircle variable region fragments of 330 bp were amplified. The PCR reactions were of 100 μl total volume; 15 μl was loaded on a 2% agarose gel. The M lane shows DNA Hae III fragments of ϕX174 RF DNA as size markers. Positive and negative controls are 1 pg kDNA and no kDNA, respectively, in the PCR reaction.

Animal biopsy material was also dissolved and stored in GE. Heart tissue obtained from infected and uninfected mice was washed with saline solution and stored in GE buffer. The tissue was dissolved by incubation in GE at 37°C for two days with occasional vigorous mixing. The dissolved tissue was processed as described in Examples 1-4. Specific PCR amplification of minicircle sequences was observed from the heart tissue lysates of the infected mouse but not from uninfected controls. These results indicate that the method could be used for autopsy specimens to detect Chagas' disease.

ADVANTAGES OF THE INVENTION

The method of the present invention has a wide variety of potential applications in the diagnosis of bacterial, parasitic, and viral diseases. It is particularly useful for the collection of specimens from patients in the field, in clinics and under other conditions in which storage conditions may be less than optimal. It is particularly adapted to the diagnosis of parasitic diseases caused by kinetoplastid trypanosomes, such as T. cruzi, the causative agent of Chagas' disease, because the catenated kinetoplast minicircle DNA characteristic of this organism is efficiently cleaved to linear fragments of a size suitable for amplification by a sequence-specific primer-based amplification technique such as PCR. The method of the invention is simple to

carry out, rapid, and highly effective in detecting small quantities of DNA specific to infectious agents. In addition, the infectivity of biological samples containing infectious agents is reduced by application of the method of the invention.

Although the present invention has been described in considerable detail with reference to certain preferred versions thereof, other versions, modifications and variations of the invention as set forth above may be made without departing from the spirit and scope of the present invention. Therefore, the invention is limited only by the terms of the appended claims.

We claim:

1. A method for isolating biologically active DNA from a biological sample having DNA-containing structures comprising:

(a) contacting a biological sample containing DNA-containing structures with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse DNA-containing structures in the sample and a chelating agent to preserve the DNA from degradation to form a mixture of the biological sample and the lysis and storage buffer;

(b) incubating the mixture formed in step (a) with a metal-containing chemical nuclease that cleaves the DNA to form DNA fragments; and

(c) purifying the DNA fragments.

2. The method of claim 1 wherein the DNA in the DNA-containing structures is present in the form of catenated closed circles.

3. The method of claim 2 wherein the DNA is DNA associated with a parasite.

4. The method of claim 3 wherein the parasite is a kinetoplastid parasite.

5. The method of claim 4 wherein the parasite is Trypanosoma cruzi.

6. The method of claim 1 wherein the chemical nuclease is selected from the group consisting of 1,10-phenanthroline-copper complex, derivatives of ferrous EDTA, metalloporphyrins and octahedral metal complexes of 4,7-diphenyl-1,10-phenanthroline.

7. The method of claim 6 wherein the chemical nuclease is 1,10-phenanthroline-copper complex.

8. The method of claim 7 wherein cleavage of the nuclease is stopped by a copper chelating agent to control the size of said DNA fragments.

9. The method of claim 1 wherein the biological sample is mammalian blood.

10. The method of claim 9 wherein the mammalian blood is human blood.

11. The method of claim 1 wherein the non-amphipathic chaotropic salt is a guanidinium salt selected from the group consisting of guanidinium chloride and guanidinium thiocyanate.

12. The method of claim 11 wherein the guanidinium salt is guanidinium chloride.

13. The method of claim 12 wherein the guanidinium chloride is present in at least about 3 molar concentration in the mixture of the biological sample and lysis and storage buffer.

14. The method of claim 1 wherein the chelating agent is EDTA.

15. The method of claim 14 wherein the EDTA is present in at least about 0.1 molar concentration in the mixture of the biological sample and lysis and storage buffer.

16. The method of claim 1 further comprising the step of:

(d) amplifying the DNA by a sequence-specific method employing at least two primers that hybridize to the DNA fragments.

17. The method of claim 16 further comprising the step of:

(e) detecting disease associated with the presence in an organism of the DNA subjected to amplification by identifying the DNA.

18. The method of claim 17 wherein the DNA is identified using DNA hybridization.

19. The method of claim 1 wherein the DNA encodes a genetic marker of the organism from which the biological sample was isolated.

20. The method of claim 1 wherein the DNA is associated with a virus.

21. A method for detecting a disease caused by a kinetoplastid parasite comprising the steps of:

(a) contacting a biological sample from a patient suspected of having a disease caused by a kinetoplastid parasite, with a lysis and storage buffer comprising a non-amphipathic chaotropic salt sufficient to lyse cells in the sample and a chelating agent to preserve the DNA in the cells from degradation to form a mixture of the biological sample and the lysis and storage buffer;

(b) incubating the mixture obtained in step (a) with a metal-containing chemical nuclease to linearize catenated closed circular kinetoplast DNA to form kinetoplast DNA fragments;

(c) purifying the DNA fragments to form purified kinetoplast DNA fragments suitable for amplification;

(d) amplifying the purified kinetoplast DNA fragments by a sequence-specific method employing at least two primers capable of hybridizing to the linearized DNA to form amplified kinetoplast DNA; and

(e) detecting disease by identifying the presence of the amplified kinetoplast DNA corresponding to the primers and thus having sequences specific for kinetoplast DNA of the parasite.

22. The method of claim 21 wherein the biological sample is mammalian blood.

23. The method of claim 22 wherein the mammalian blood is human blood.

24. The method of claim 21 wherein the non-amphipathic chaotropic salt is a guanidinium salt selected from the group consisting of guanidinium chloride and guanidinium thiocyanate.

25. The method of claim 24 wherein the guanidinium salt is guanidinium chloride.

26. The method of claim 21 wherein the chelating agent is EDTA.

27. The method of claim 21 wherein the chemical nuclease is selected from the group consisting

of 1,10-phenanthroline-copper complex, derivatives of ferrous EDTA, metalloporphyrins, and octahedral metal complexes of 4,7-diphenyl-1,10-phenanthroline.

28. The method of claim 27 wherein the chemical nuclease is the 1,10-phenanthroline-copper complex.

29. The method of claim 21 wherein the kinetoplastid parasite is Trypanosoma cruzi.

30. The method of claim 21 wherein the primers hybridize to conserved regions in T. cruzi kinetoplast minicircular DNA.

31. A method for isolating and storing DNA from a cell-containing biological sample comprising the steps of:

(a) contacting a biological sample containing DNA present in cells with a lysis and storage buffer in order to liberate the DNA from the cells, the lysis and storage buffer comprising:

(i) a concentration of a non-amphipathic chaotropic salt sufficient to lyse cells in the biological sample when the lysis buffer is contacted with the biological sample; and

(ii) a concentration of a chelating agent sufficient to preserve the DNA from degradation when the lysis buffer is contacted with the biological sample; and

(b) storing the liberated DNA in the mixture at a temperature of below about 65°C.

32. The method of claim 31 wherein the non-amphipathic chaotropic salt is guanidinium chloride present in at least about 3 molar concentration in the mixture of the biological sample and the lysis and storage buffer and the chelating agent is EDTA present in at least about 0.1 molar concentration in the mixture of the biological sample and the lysis buffer.

33. The method of claim 31 wherein the storage temperature is below about 4°C in order to preserve the DNA for at least about one year.

34. The method of claim 31 wherein the DNA is present in the cells in the form of catenated closed circles.

35. A method for detecting a disease caused by the kinetoplastid parasite T. cruzi comprising the steps of:

(a) mixing a mammalian blood sample suspected of containing kinetoplastid DNA from T. cruzi with about an equal volume of lysis and storage buffer comprising 6

molar guanidinium chloride and 0.2 molar EDTA, pH 8.0 to form a mixture and storing the mixture at room temperature for at least 24 hours;

(b) incubating the mixture obtained in step (a) with the chemical nuclease 1,10-phenanthroline-copper complex to cleave the kinetoplast DNA to form linearized kinetoplast DNA;

(c) purifying the linearized kinetoplast DNA to form purified linearized kinetoplast DNA by:

(i) deproteinization with a phenol-chloroform mixture;

(ii) ethanol precipitation with a glycogen carrier; and

(iii) filtration through a microconcentrator;

(d) amplifying the purified linearized kinetoplast DNA by a sequence-specific method employing at least two primers capable of hybridizing to conserved regions of the catenated closed circular kinetoplast DNA of T. cruzi; and

(e) detecting disease caused by T. cruzi by identifying the presence of amplified DNA corresponding to the primers and thus having sequences specific for catenated closed circular kinetoplast DNA of T. cruzi by hybridization to the primers.

36. The method of claim 35 wherein the linearized DNA comprises fragments of from about 1 kb to about 1.5 kb in length.

37. The method of claim 34 wherein the sequence-specific amplification method is the polymerase chain reaction method.

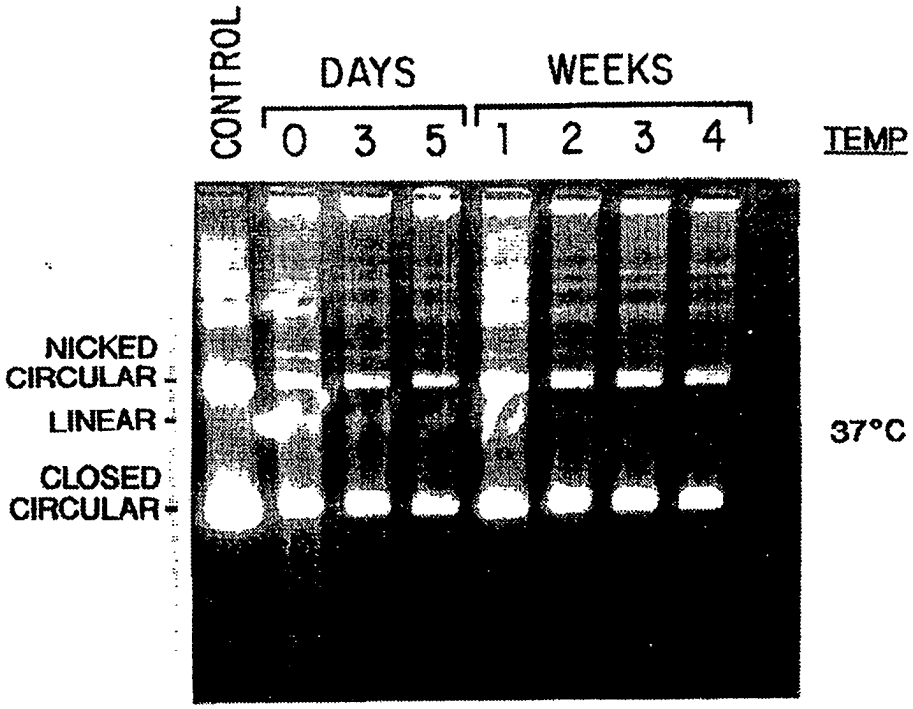

CONTROL
DAYS
WEEKS
0 3 5 1 2 3 4
TEMP
NICKED CIRCULAR
LINEAR
CLOSED CIRCULAR
37°C

FIG. 1A

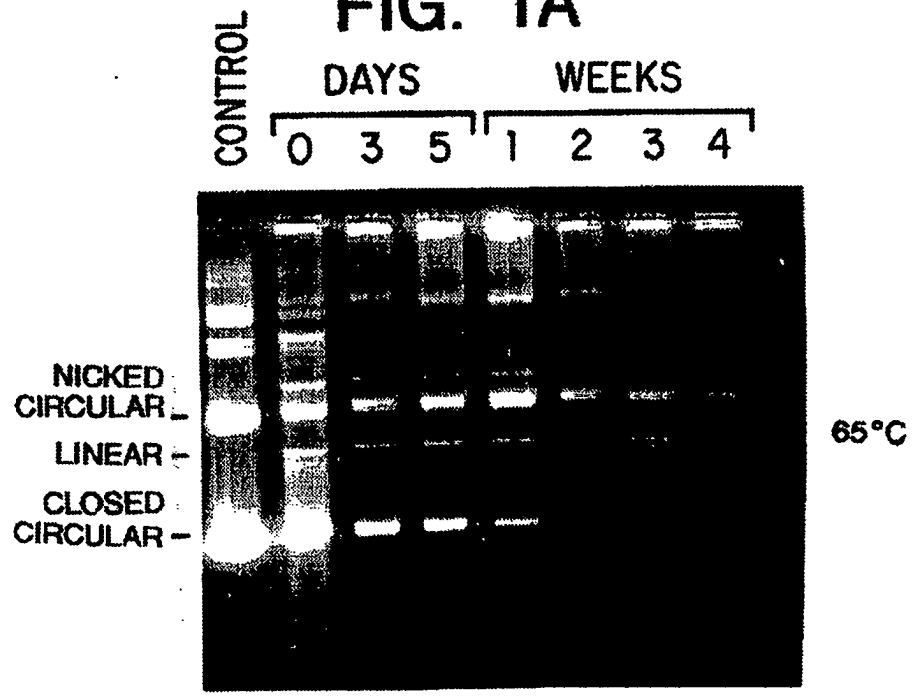

CONTROL
DAYS
WEEKS
0 3 5 1 2 3 4
NICKED CIRCULAR
LINEAR
CLOSED CIRCULAR
65°C

FIG. 1B

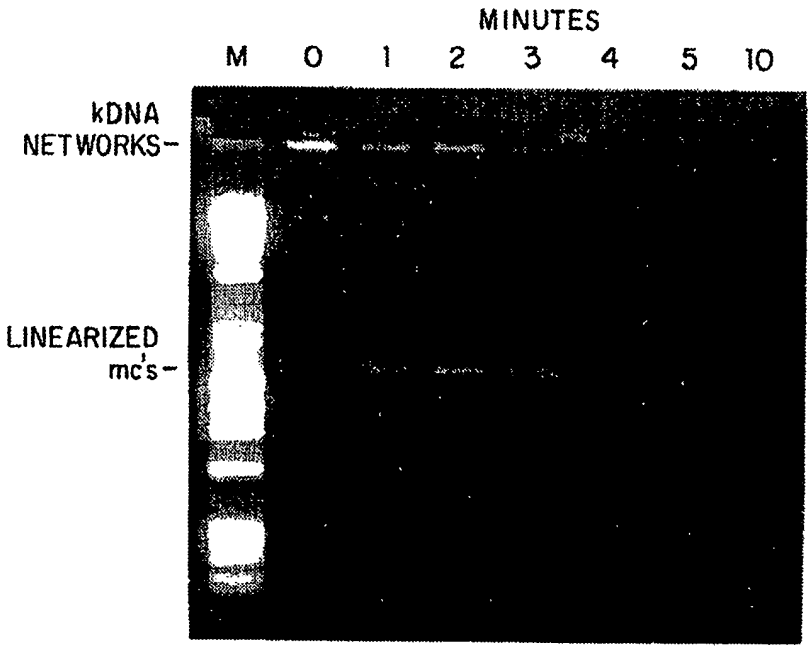


FIG. 2

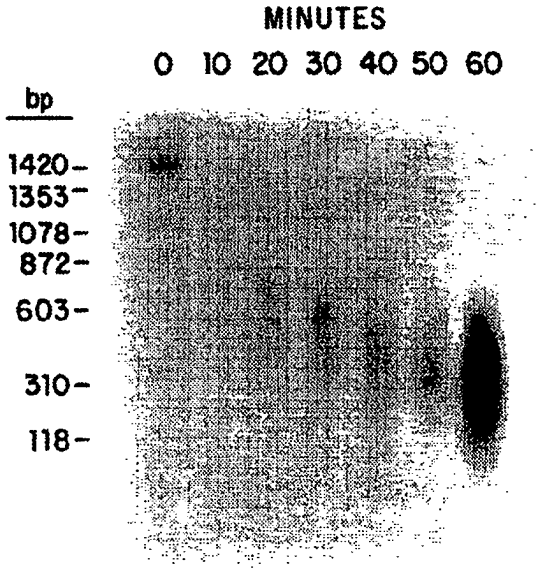


FIG. 3

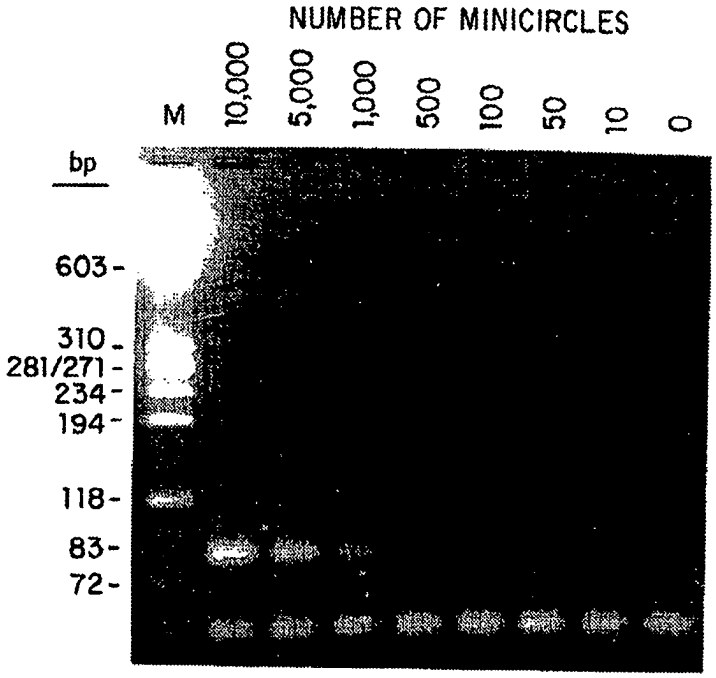
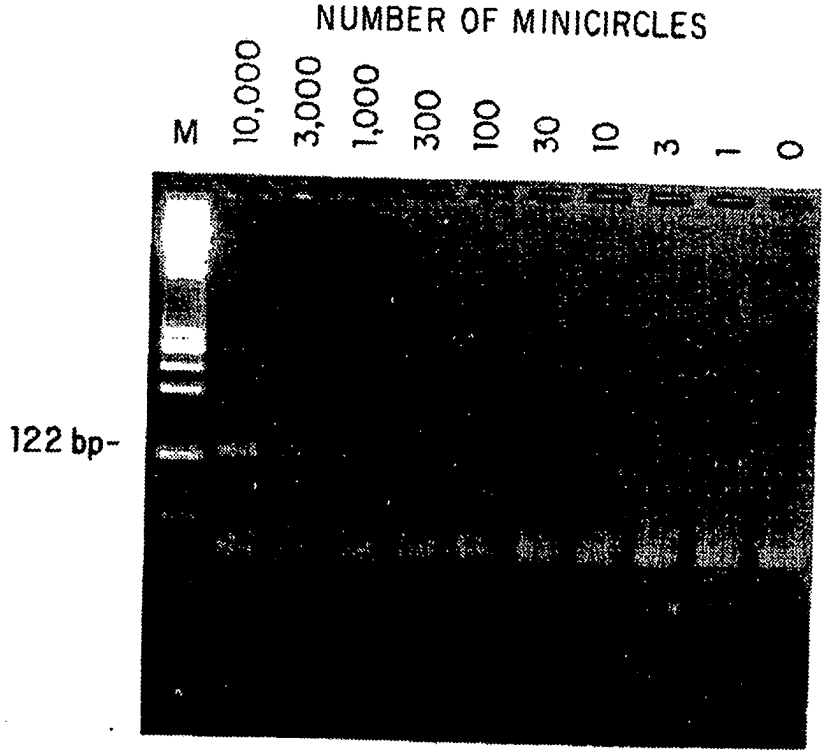

NUMBER OF MINICIRCLES
M
10,000
5,000
1,000
500
100
50
10
0
bp
603-
310-
281/271-
234-
194-
118-
83-
72-

FIG. 4A


NUMBER OF MINICIRCLES
M
10,000
3,000
1,000
300
100
30
10
3
1
0
122 bp-

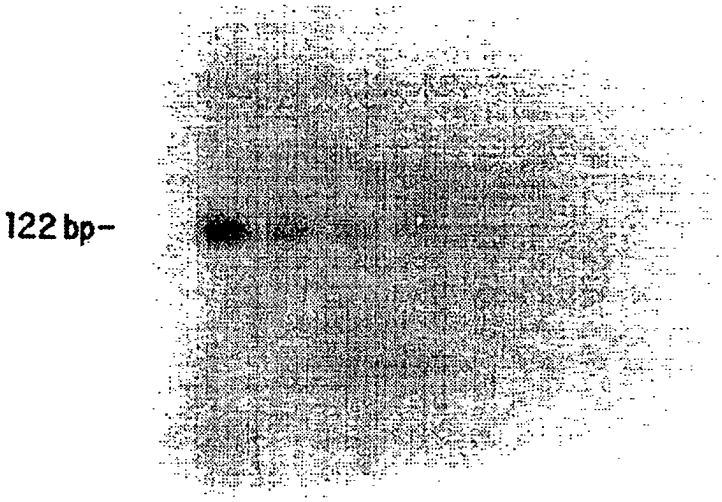

122 bp-

FIG. 4B

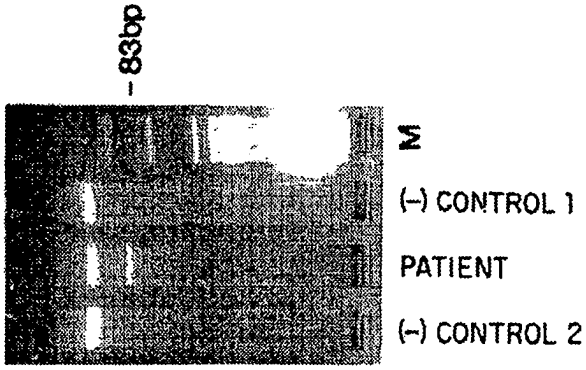

- 83bp
M
(-) CONTROL 1
PATIENT
(-) CONTROL 2

FIG. 5A

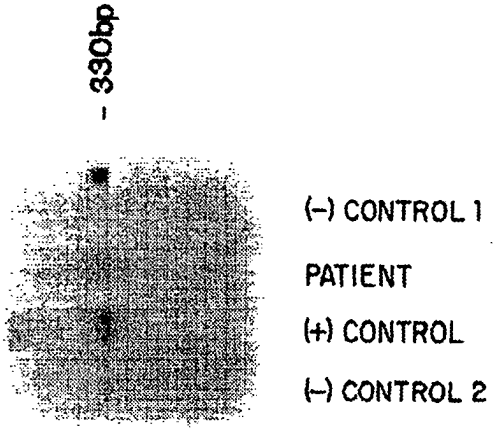

- 330bp
(-) CONTROL 1
PATIENT
(+) CONTROL
(-) CONTROL 2

FIG. 5B

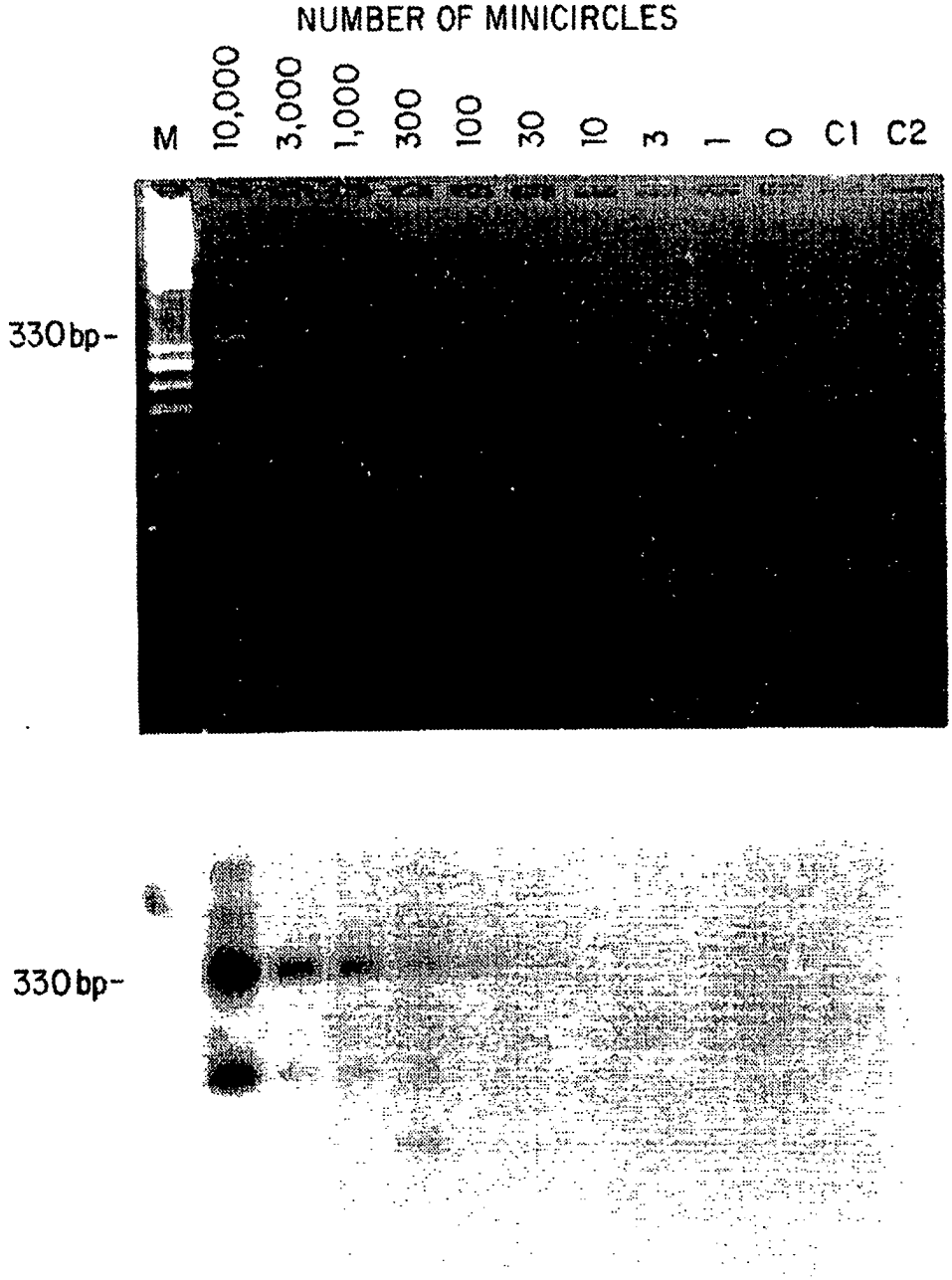

NUMBER OF MINICIRCLES
M
10,000
3,000
1,000
300
100
30
10
3
1
0
C1
C2
330bp-
330bp-

FIG. 5C

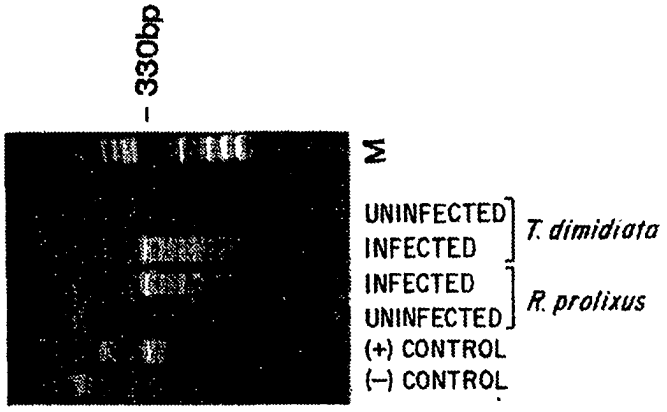

330bp
M
UNINFECTED
INFECTED
T. dimidiata
INFECTED
UNINFECTED
R. prolixus
(+) CONTROL
(–) CONTROL

FIG. 6

INTERNATIONAL SEARCH REPORT

In..national application No.
PCT/US92/05477

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 19/34; C12Q 1/68
US CL :435/91, 6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CHEMICAL ABSTRACTS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular and Biochemical Parasitology, Volume 33, issued 1989, N. R. Sturm et al., "Sensitive detection and schizodeme classification of Trypanosoma cruzi cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease", pages 205-214, ENTIRE DOCUMENT.	1-37
Y	Experimental Parasitology, Volume 71, issued 1990, M. R. Rodgers et al., "Amplification of Kinetoplast DNA as a Tool in the Detection and Diagnosis of Leishmania", pages 267-275, especially page 273, paragraph bridging the left- and right-hand columns.	1-37
Y,P	US, A, 5,130,423 (Van Ness et al.) 14 July 1992, col. 3, lines 53-59.	1-37
Y	Annual Review of Biochemistry, Volume 59, issued 1990, D. S. Sigman et al., "CHEMICAL NUCLEASES: NEW REAGENTS IN MOLECULAR BIOLOGY", pages 207-236, entire document.	1-37

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 SEPTEMBER 1992

Date of mailing of the international search report

01 OCT 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

JAMES KETTER

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★